REMARKS

Pending claims

Claims 1-20 were originally filed in this application. By this Amendment, claims 1-20 have been cancelled and substituted with new claims 21-34. New claims 21 and 33 basically correspond to originally filed claims 1, 2 and 15. New claim 22 basically corresponds to originally filed claims 3-4 (it is submitted that this claim falls within the election of the Restriction Requirement and contains the elected SEQ ID NO). New claims 23-24 basically correspond to originally filed claim 12 (it is submitted that these claims fall within the election of the Restriction Requirement and contain the elected SEQ ID NO). New claims 24 and 26 basically correspond to originally filed claims 13 and 14, respectively (it is submitted that these claims fall within the election of the Restriction Requirement and contain the elected SEQ ID NO). New claim 25 is directed to a transgenic organism comprising the recombinant polynucleotide of claim 22. New claim 27 basically corresponds to originally filed claim 16. New claims 28-29 basically correspond to originally filed claims 9, 10 and 11. New claim 29 basically corresponds to originally filed claims 9, 10 and 11 but is directed to SEQ ID NO:70 (it is submitted that this claim falls within the election of the Restriction Requirement and contains the elected SEQ ID NO). New claims 30-32 basically correspond to originally filed claims 7-8 but are directed to SEQ ID NO:70. New claim 33 basically corresponds to originally filed claim 15. New claim 34 basically corresponds to originally filed claim 19.

Please note that the originally filed claims directed to the agonist, antagonist and methods using the claimed agonist or antagonist are not contained in the set of new claims. Applicants expressly state that these claims are not being pursued in order to expedite prosecution of the new claims and **not** for reasons related to patentability, and are in fact fully supported by the specification as filed. Applicants expressly reserve the right to reinstate these claims or to add other claims during prosecution of this application or a continuation or divisional application. Applicants expressly do not disclaim the subject matter of any invention disclosed herein which is not set forth in the instantly filed new claims.

Restriction Requirement

In the Restriction Requirement, the Examiner requested Applicants to elect one of the following inventions:

Groups 1-65 (claims 1, 2 and 15) drawn to polypeptides and pharmaceutical compositions comprising said polypeptides with the sequence of SEQ ID NO:1-65, respectively.

Groups 66-130 (claims 3-6 and 12-14) drawn to polynucleotides encoding polypeptides with the sequence of SEQ ID NO:1-65, respectively, vectors containing said polynucleotides, host cells containing said vectors and a method of producing polypeptides utilizing the aforementioned polynucleotides.

Groups 131-195 (claims 9-11) drawn to polynucoetides with the sequence of SEQ ID NO:66-130, respectively.

Groups 196-260 (claim 16) drawn to antibodies that bind polypeptides with the sequence of SEQ ID NO:1-65, respectively.

Groups 261-325 (claim 1) drawn to agonists of polypeptides with the sequence of SEQ ID NO:1-65, respectively.

Groups 391-455 (claim 19) drawn to methods of treating a disorder associated with the increased [*sic*] [*should be decreased*] expression or activity of HTRM utilizing pharmaceutical compositions comprising polypeptides with the sequence of SEQ ID NO:1-65, respectively.

Groups 456-520 (claim 20) drawn to methods of treating a disorder associated with the increased expression or activity of HTRM utilizing agonists to polypeptides with the sequence of SEQ ID NO:1-65, respectively.

Groups 521-585 (claims 9-11 [*sic*] [*should be claims 7-8*]) drawn to methods of detecting polynucleotides using polynucleotides that encode polypeptides with the sequence of SEQ ID NO:1-65, respectively, as a probe.

Applicants hereby elect, with traverse, to prosecute Group 70, which includes and is drawn to new claims 22-24, 26 and 29. Applicants reserve the right to prosecute the subject matter of non-elected claims in subsequent divisional applications.

In the Restriction Requirement, the Examiner further requested Applicants to elect a single SEQ ID NO.

Applicants hereby elect, with traverse, to prosecute SEQ ID NO:70, which includes and is drawn to new claims 22-24, 26 and 29. Applicants reserve the right to prosecute the subject matter of non-elected SEQ ID NOs in subsequent divisional applications.

Applicants traverse this Restriction Requirement on several grounds.

First, Applicants traverse the Restriction Requirement as between the claims of Group 70 (original claims 3-6 and 12-14; new claims 22-24, 26 and 29) and Group 525 (i.e. original claims 7-8; new claims 30-32, drawn to methods of detecting a target polynucleotide having a sequence of a polynucleotide of claim 29). The method claims are directed to a method which depends on knowing the sequence of the polynucleotide of claim 29. Therefore, a search of the claimed polynucleotides would substantially overlap examination of method claims 30-32 and would not be an undue burden on the Examiner.

Second, Applicants traverse the Restriction Requirement as between the claims of Group 70 and Group 5 (drawn to polypeptides related to SEQ ID NO:70). Many of the elected claims of Group 70 are directed specifically to polynucleotides encoding the claimed polypeptides of Group 5, and thus it is presumed that a proper search for the claimed polynucleotides would include the polypeptides which they encode. Therefore, it is submitted that it would not be a substantial burden on the Examiner to use the results of the necessary polynucleotide search to examine the polypeptide claims.

Third, Applicants traverse the Restriction Requirement as between the Group 5 and Group 200 (drawn to the polypeptides and antibodies to the polypeptide, respectively), and hence Group 70. The claims of these groups could be examined at the same time, also without undue burden on the Examiner. A search of the prior art to determine the novelty of the antibodies would substantially overlap with a search of the claims directed to the polypeptides. Thus, Applicants submit that examining the prior art for the polypeptides together with the antibodies would involve substantially the same subject matter and would not impose an undue burden on the Examiner.

Accordingly, as submitted above, a search of the claimed polynucleotides of Group 70 would include the claimed encoded polypeptides of Group 5. Therefore, it is submitted that it would not be a

Table 1

Protein SEQ ID NO:	Nucleotide SEQ ID NO:	Clone ID	Library	Fragments
1	66	001106	U937NOT01	001106 (U937NOT01), 1291142 (BRAINOT11), 2590425 (LUNGNOT22), 1300570 (BRSTNOT07)
2	67	004586	HMC1NOT01	004586 (HMC1NOT01), 3889843 (BRSTTUT16), 1432988 (BEPINON01), 788995 (PROSTUT03), 1605475 (LUNGNOT15)
3	68	052927	FIBRNOT01	052927 (FIBRNOT01), 2518848 (BRAITUT21), 3520218 (LUNGNON03), 086878 (LIVRNOT01)
4	69	082843	HUVESTB01	082843 (HUVESTB01), 4008105 (ENDCNOT04), 2083528(UTRSNOT08), 2345764 (TESTTUT02), 3771780 (BRSTNOT25), 190782 (CONNTUT01), 2206259 (SPLNFET02), 2509193 (CONUTUT01)
5	70	322349	EOSIHET02	322349 (EOSIHET02), 3686018 (HEAANOT01), 1853592(LUNGFET03), 815966 (OVARTUT01), 1505002 (BRAITUT07), 1511883 (LUNGNOT14), 2232826 (PROSNOT16)
6	71	397663	PITUNOT02	397663 (PITUNOT02), 491141 (HNT2AGT01), 3809879(CONTTUT01) 3562349 (SKINNOT05), 1518413 (BLADTUT04), 3600151 (DRGTNOT01), 2474103 (THP1NOT03), 2105304 (BRAITUT03),2187330 (PROSNOT26), 1781572 (PGANNON02), 2056258 (BEPINOT01), 1888065 (BLADTUT07)
7	72	673766	CRBLNOT01	673766 (CRBLNOT01), 2494421 (ADRETUT05), 3267748 (BRAINOT20) 2194042 (THYRTUT03), 3186455 (THYMNON04), 1712236 (PROSNOT16) 1844092 (COLNNOT08), 1602283 (BLADNOT03), 033357 (THP1NOB01), 1995828 (BRSTTUT03), 1485594 (CORPNOT02)
8	73	1504753	BRAITUT07	1504753 (BRAITUT07), 633939 (NEUTGMT01), 2741379 (BRSTTUT14), 2959661 (ADRENOT09), 3483904 (KIDNNOT31), 999401 (KIDNTUT01), 1965504 (BRSTNOT04), 588535 (UTRSNOT01)
9	74	1760185	PITUNOT03	1760085 (PITUNOT03), 1914471 (PROSTUT04), 836831(PROSNOT07), 729798 (LUNGNOT03), 1290847 (BRAINOT11), 1492387 (PROSNON01),1368472 (SCORNON02)

substantial burden on the Examiner to use the results of the necessary polynucleotide search to examine the polypeptide together with the antibody claims of Group 200.

In support of this second and third grounds is the attached Giang and Cravatt article. The attached article discloses a very similar polynucleotide, a very similar polypeptide encoded by this very similar polynucleotide, and the making of antibodies to this very similar polypeptide. Accordingly, this article demonstrates that examining the prior art for the polynucleotides together with the polypeptides and antibodies would involve substantially the same subject matter/sources and would not impose an undue burden on the Examiner.

In addition, Applicants submit that new claim 25, drawn to a transgenic organism comprising the recombinant polynucleotide of claim 23 belongs within the elected claims of Groups 70. This claim is directed to a product that contains the claimed recombinant polynucleotide of claim 23 to be searched by the Examiner. Therefore, a search of the claimed recombinant polynucleotide of claim 23 would substantially overlap examination of a transgenic organism of claim 25 and would not be an undue burden on the Examiner.

Therefore, it is respectfully submitted that, upon searching and examining polynucleotides encoding the polypeptides relating to SEQ ID NO:5 and finding no prior art over which they can be rejected, the search should be extended to include the polypeptides encoded by SEQ ID NO:70 and antibodies which specifically bind to SEQ ID NO:5.

Rejoinder

Applicants traverse on the grounds that the Examiner could also examine new claims 30-32, drawn to methods of detecting a target polynucleotide having a sequence of a polynucleotide of claim 38. The Examiner's attention is directed to the Commissioner's Notice in the Official Gazette of March 26, 1996, entitled "Guidance on Treatment of Product and Process Claims in Light of *In re Ochiai, In re Brouwer* and 35 U.S.C. § 103(b)" which sets forth the rules, upon allowance of product claims, for rejoinder of process claims covering the same scope of products. Therefore, upon allowance of any of the claims within Group 70, *i.e.* new claims 22-24, 26 and 29, the new method claims 30-32, which depend therefrom, should be rejoined and examined.

Applicants believe that no fee is due with this communication. However, if the USPTO determines that a fee is due, the Commissioner is hereby authorized to charge Deposit Account No. 09-0108.

Respectfully submitted,

INCYTE CORPORATION

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VERSION WITH MARKINGS TO SHOW CHANGES MADE

IN THE SPECIFICATION:

The paragraph beginning at line 3 of page 1 has been inserted as follows:

This application is the National Stage of International Application No. PCT/US99/09935, filed on May 4, 1999, which claims the benefit under 35 U.S.C. § 119(e) of U.S. Provisional Application Serial No. 60/084254, filed May 5, 1998; U.S. Provisional Application Serial No. 60/095827, filed August 7, 1998; and U.S. Provisional Application Serial No. 60/102745, filed October 2, 1998, the contents all of which are hereby incorporated herein by reference.

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 273, No. 12, Issue of March 20, pp. 6595–6598, 1998

Communication

A Second Mammalian *N*-Myristoyltransferase*

(Received for publication, December 23, 1997, and in revised form, January 21, 1998)

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N-terminal myristoylation is a cotranslational lipid modification common to many signaling proteins that often serves an integral role in the targeting and/or function of these proteins. Myristoylation is catalyzed by an enzyme activity, N-myristoyltransferase (NMT), which transfers myristic acid from myristoyl coenzyme A to the amino group of a protein's N-terminal glycine residue. While a single human NMT cDNA has been isolated and characterized (hNMT-1), biochemical evidence has indicated the presence of several distinct NMTs *in vivo*, often varying in either apparent molecular weight and/or subcellular distribution. We now report the cloning and characterization of a second, genetically distinct human NMT (hNMT-2), as well as the isolation of the respective mouse NMT homologue for each human enzyme. The mouse and human versions of each NMT are highly homologous, displaying greater than 95% amino acid sequence identity. Comparisons between the NMT-1 and NMT-2 proteins revealed reduced levels of sequence identity (76–77%), indicating that NMT-1 and NMT-2 comprise two distinct families of *N*-myristoyltransferases. Transient transfection of either the hNMT-1 or hNMT-2 cDNA into COS-7 cells resulted in the expression of high levels of NMT enzyme activity. Both hNMT-1 and hNMT-2 were found to myristoylate several commonly studied peptide substrates with similar, but distinguishable, relative selectivities. Western analysis revealed that while hNMT-2 appeared as a single 65-kDa protein in transfected COS-7 cells, hNMT-1 was processed to provide four distinct protein isoforms ranging from 49 to 68 kDa in size. Collectively, these studies demonstrate a heretofore unappreciated level of genetic complexity underlying the enzymology of N-terminal myristoylation and suggest that the specific inhibition or regulation of either NMT *in vivo* may in turn allow for the selective control of particular myristoylation-dependent cellular functions.

The cotranslational modification of proteins with myristic acid serves to regulate both protein function and localization (1, 2). Most myristoylated proteins are acylated through an amide linkage to their N-terminal glycine residues, a reaction catalyzed by the enzyme, *N*-myristoyltransferase (NMT)[1] (EC 2.3.1.97) (3–5). Myristoylation has proven essential to the biological activity of many mammalian, viral, and fungal proteins. In particular, the transformation potential of the protein tyrosine kinase, $p60^{src}$, is entirely dependent on myristoylation, as nonmyristoylated forms of $p60^{src}$ fail to bind cellular membranes and are transformation defective (6). Similarly, nonmyristoylated forms of endothelial nitric-oxide synthase are not properly localized to the Golgi apparatus and plasmalemmal caveolae, resulting in marked reductions in stimulated nitric oxide production (7, 8). The dependence of viral infectivity on myristoylation is exemplified by the observation that inhibiting the myristoylation of the human immunodeficiency virus type I GAG precursor protein promotes the production of noninfectious viral particles (9).

Genetic studies have shown that the NMT gene is essential for the viability of the yeast, *Saccharomyces cerevisiae* (10), and the pathogenic fungi, *Candida albicans* (11) and *Cryptococcus neoformans* (12). Accordingly, inhibitors of *C. albicans* NMT have proven to be potent antifungal agents (13). In mammalian systems, NMT activity has been shown to increase in colorectal tumors (14, 15), leading to the proposal that NMT could serve as a target for anticancer therapies (16). In this regard, one speculated mechanism for the antitumor activity of the natural product fumagillin is through indirectly preventing protein myristoylation (17). Fumagillin has been shown to inhibit the methionine aminopeptidase, MetAP-2, an enzyme that cleaves the N-terminal methionine from newly synthesized proteins (17), a process required for the exposure of N-terminal glycine residues of NMT protein substrates.

A single human NMT cDNA has been isolated and characterized (18, 19), and subsequent failures to identify homologous human cDNAs has led some to speculate that NMT activity *in vivo* is likely derived from a single gene (20). However, biochemical studies have repeatedly indicated the presence of multiple distinct protein forms of NMT *in vivo*, often varying in either molecular size and/or subcellular distribution (2, 21–23). Whether all of these NMT forms are derived from a single gene or from multiple NMT genes has remained unclear (2). We now report the isolation and characterization of a second distinct NMT cDNA from a human liver library, as well as the cloning of the respective mouse homologue for each of the two human NMTs. For the sake of clarity, we will hereafter refer to the originally characterized human NMT as hNMT-1 and the human NMT described in the present study as hNMT-2.

EXPERIMENTAL PROCEDURES

Cloning of Human and Mouse NMT cDNAs—PCR primers based on the sequence of expressed sequence tag (EST) AA036845 were designed for the amplification of a 550-base pair portion of the hNMT-2 cDNA from a human liver 5′Stretch Plus cDNA library (CLONTECH): Primer 1, 5′-GCGAATTCAACATCCACACAGAGACGCCC-3′; Primer 2, 5′-GCGAATTCTCTGTAACTTCTACTTAGTCC-3′. This amplified DNA was used as a probe to screen human and mouse liver 5′Stretch Plus cDNA libraries according to the manufacturer's guidelines. Four positive hu-

* This work was supported by the Skaggs Institute for Chemical Biology. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF043324 (hNMT-1), AF043325 (hNMT-2), AF043326 (mNMT-1), and AF043327 (mNMT-2).

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[1] The abbreviations used are: NMT, *N*-myristoyltransferase; hNMT, human *N*-myristoyltransferase; mNMT, mouse *N*-myristoyltransferase; EST, expressed sequence tag; GST, glutathione *S*-transferase; PKA, cAMP-dependent protein kinase; PCR, polymerase chain reaction; kb, kilobase pair(s).

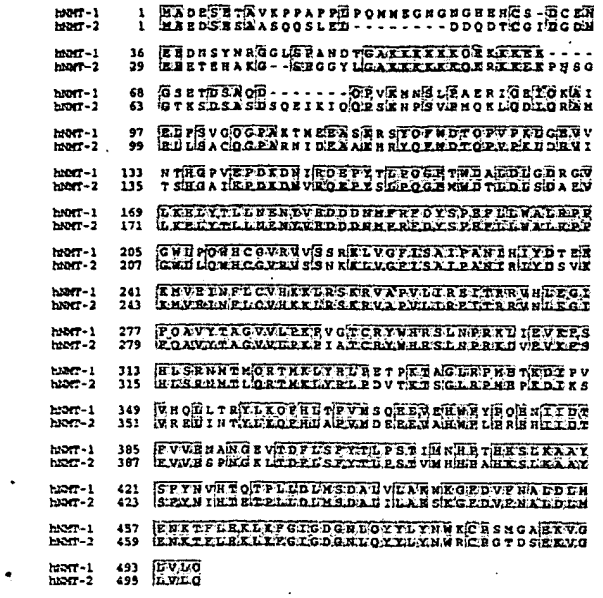

FIG. 1. **Comparison of the deduced amino acid sequences from human NMT-1 and NMT-2 cDNAs.** Shared sequence identities between NMT-1 and NMT-2 are *shaded*.

man clones and six positive mouse clones were isolated from screenings of 3.2×10^5 and 9.6×10^5 plaques, respectively. A 2.85-kb human clone and a 1.9-kb mouse clone contained the presumed complete coding sequence for hNMT-2 and mNMT-2, respectively. The hNMT-1 cDNA was isolated by PCR with primers based on the reported GenBank™ sequence (accession number AF020500): Primer 1, 5′-GCAAGCTTCGCTGCCGCAGATGATGG-3′, Primer 2, 5′-GCGAATTCAGTTCTGCTCCCTTTGCC-3′. This hNMT-1 cDNA was then used as a probe to screen both human brain and mouse liver cDNA libraries (CLONTECH; 3.2×10^5 plaques per screening). Six positive human clones and eight positive mouse clones were identified. A 4.4-kb human clone and 1.6-kb mouse clone encoded the presumed complete coding sequence for hNMT-1 and mNMT-1, respectively. All reported cDNAs were cloned into pBluescript II SK(+) and sequenced completely in both directions. NMT protein sequence comparisons were conducted using the DNASTAR MegAlign program (Clustal method), and percentage sequence identities were calculated from sequence alignments after excluding the identified gaps. For Northern analysis, cDNA probes specific for the NMT-1 and NMT-2 genes were generated from the 5′ most 400 base pairs of each cDNA (the least homologous region between NMT-1 and NMT-2).

Expression of hNMTs in COS-7 Cells—The hNMT-1 and hNMT-2 cDNAs were cloned into the eukaryotic expression vector, pcDNA3, and transiently transfected into COS-7 cells as described previously (24), with the exception that 1.25 μg of the hNMT-1 cDNA was used per transfection to reduce the amount of hNMT-1 expression to levels comparable with hNMT-2 expression (as judged by NMT enzyme activities). Transfected cells were harvested by trypsinization, washed twice with Hepes buffer (12.5 mM Hepes, pH 8.0, 1 mM EDTA, 100 mM NaCl), and Dounce-homogenized in Hepes buffer containing 1 × complete protease inhibitors (Boehringer Mannheim) on ice. The homogenized cell extracts were then sonicated briefly (5 s), assayed for protein content (D_c protein assay kit, Bio-Rad), and used for enzyme assays, Western blotting, and cell fractionation experiments (25).

Generation of Anti-NMT Antibodies—The hNMT-1 cDNA was cloned into the pGEX-4T-3 vector (Amersham Pharmacia Biotech) for generation of a GST-hNMT-1 fusion protein (residues 89–496 of hNMT-1) according to manufacturer's guidelines. Rabbit polyclonal antibodies generated against the GST-NMT-1 fusion protein were affinity-purified by first removing GST-cross-reactive antibodies and then isolating antibodies that bound the GST-hNMT-1 protein. These antibodies were used in standard Western blotting procedures and recognized both the hNMT-1 and hNMT-2 proteins.

N-Myristoyltransferase Assays—*N*-Myristoyltransferase activity was monitored using the methods employed by Towler and Glaser (26). [^{3}H]Myristoyl-CoA (0.75 μCi; 52 Ci/mmol; 0.3 μM; Amersham Pharmacia Biotech) was added to a mixture of COS-7 total cell protein (7.5 μg) and peptide substrate (200 μM) in a reaction buffer of 30 mM Tris-HCl, pH 7.5 with 0.5 mM EDTA, 0.5 mM EGTA, 1.0% (v/v) Triton X-100, and 4.5 mM β-mercaptoethanol (total reaction volume of 50 μl; reaction

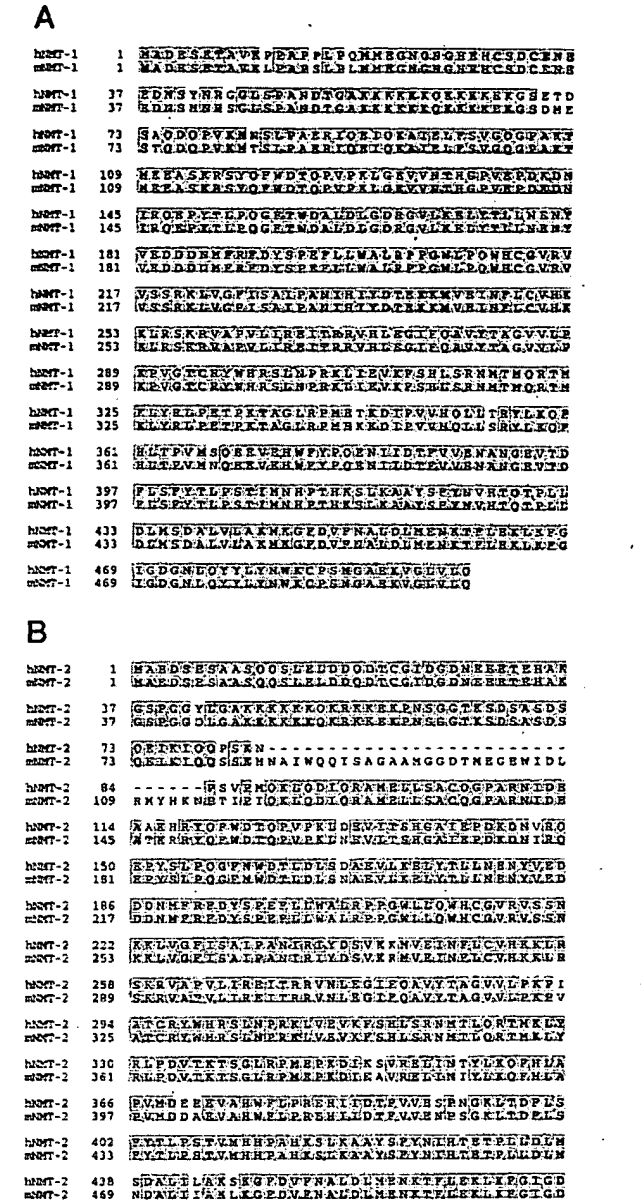

FIG. 2. **Comparisons of the deduced amino acid sequences from human and mouse NMT cDNAs.** *A*, comparison of the human and mouse NMT-1 proteins (hNMT-1 and mNMT-1, respectively); *B*, comparison of the human and mouse NMT-2 proteins (hNMT-2 and mNMT-2, respectively). Shared sequence identities between the human and mouse NMTs are *shaded*.

buffer from King and Sharma (27)). The reaction was allowed to proceed for 10 min at 25 °C, then quenched with 50 μl of methanol followed by 5 μl of 100% trichloroacetic acid, placed on ice for 10 min, and spun at 10,000 × *g* for 5 min. Aliquots (25 μl) of the supernatant were analyzed by reverse-phase high pressure liquid chromatography. A myristoylated GNAAAARR peptide was synthesized as described by Towler and Glaser (26) and used as a standard to define the elution times for myristoylated peptide products. Column fractions (1 ml) were collected and counted by scintillation counting. In all cases, control reactions without peptide were also analyzed and subtracted from reactions with peptide to provide the myristoylation rates reported in Table I. Each reaction was run in triplicate with data reported as the average ± S.D. of these values. Initial rates were determined from reactions in which less than 20% myristoylated product was formed. Peptide substrates were designed based on previous work of Boutin and colleagues (28).

RESULTS AND DISCUSSION

Cloning of Human and Mouse NMTs—Intrigued by an apparent disparity between the available biochemical and genetic

TABLE I
N-Myristoyltransferase activities of hNMT-1- and hNMT-2-transfected COS-7 cells

Substrate	Mock-transfected myristoylation rate	hNMT-1-transfected myristoylation rate	hNMT-1 myristoylation rate	hNMT-2-transfected myristoylation rate	hNMT-2 myristoylation rate
	pmol · min^{-1} · mg^{-1}		%	*pmol · min^{-1} · mg^{-1}*	%
Src peptide (GSSKSKPKDP)	6.4 ± 0.2	34.7 ± 1.4	100	38.5 ± 1.6	100
PKA peptide (GNAAAARR)	1.9 ± 0.1	21.9 ± 0.7	70	17.9 ± 0.3	50
c-Abl peptide (GQQPGKVL)	1.0 ± 0.3	13.2 ± 2.7	43	9.5 ± 1.5	27
Tumor necrosis factor peptide (EEALPKKTGGPQGSR)	ND	ND		ND	

N-Myristoyltransferase activity measured in transfected COS-7 cell extracts with various potential peptide substrates. Mock-transfected COS-7 cells were transfected with the pcDNA3 vector alone. For hNMT-1 and hNMT-2, myristoylation rates with the Src peptide were considered to be 100% hNMT activity, to which other peptide myristoylation rates were compared. Absolute myristoylation rates were calculated after subtraction of control values from reactions run in the absence of peptide, with data reported as the average of three trials ± S.D. Percentile hNMT myristoylation rates were calculated after subtraction of mock transfection myristoylation rates. ND = no detectable activity.

data on *N*-myristoyltransferase enzymes, we searched the EST data base for homologues to a previously characterized human NMT cDNA (hNMT-1). Identified ESTs separated into two distinct categories, either being identical to the hNMT-1 cDNA or approximately 70–80% identical to the hNMT-1 cDNA (*e.g.* EST AA203325, AA036845, AA036785, AA364769). Moreover, the collection of homologous, but distinct, ESTs appeared to be derived from a common cDNA, leading us to conclude that a second as of yet uncharacterized NMT existed in humans. Oligonucleotide primers based on the cDNA sequence of EST AA036845 were used in the PCR to amplify a 550-base pair fragment of the novel NMT cDNA from a human liver cDNA library. This PCR product was subsequently used to isolate a 2.85-kb cDNA that encoded a putative full-length NMT (hNMT-2). Although no stop codon was identified upstream of the first encoded methionine residue, the sequence surrounding this ATG matched well with the Kozak sequence for predicted eukaryotic translation initiation sites (29). Additionally, this putative translation initiation site was shared by all four mammalian NMT cDNAs and coded for the only conserved in-frame methionine residue upstream of the previously defined NMT ribosomal targeting domain (19) (see below). Comparisons between the predicted protein sequences for hNMT-1 and hNMT-2 demonstrated that the two proteins shared 77% amino acid identity (Fig. 1). Sequence divergence was most prevalent in the N-terminal domains of the NMTs, a region of hNMT-1 dispensable for catalytic activity, but implicated in protein targeting (19, 30). In this regard, an intriguing conserved sequence was noted in this otherwise dissimilar region of the NMTs, as amino acids 54–68 of hNMT-1, GAKKKKK-KQKKKKEK, matched nearly identically amino acids 44–58 of hNMT-2, GAKKKKKKQKRKKEK. Similar stretches of positively charged residues have been identified in other proteins involved in the cotranslational processing of proteins, including *N*-methionylaminopeptidase (31), leading to the proposal that these sequence elements serve to target such enzymes to the ribosome (19).

To gain a better understanding of the potential significance of the sequence divergence between hNMT-1 and hNMT-2, their respective mouse homologues, mNMT-1 and mNMT-2, were isolated and characterized. Interestingly, the mouse and human NMTs segregated neatly into two distinct pairs of enzymes based on their homologies in primary structure (Fig. 2), with mNMT-1 and hNMT-1 sharing 97% amino acid sequence identity and mNMT-2 and hNMT-2 sharing 96% amino acid sequence identity. One notable difference between mNMT-2 and hNMT-2 was the presence in the former of an inserted stretch of 31 amino acids in the N-terminal portion of the protein (amino acids 84–114). Further examination of the mNMT-2 cDNA failed to identify consensus intron splice sites within this inserted sequence, indicating that amino acids 84–114 were a legitimate part of the mNMT-2 protein. Additionally, five independent mNMT-2 cDNA clones were isolated and one EST (W62224) identified that covered this region of the mNMT-2 sequence and each of these clones possessed this insert. A comparison between the mNMT-1 and mNMT-2 proteins revealed a reduced level of sequence identity of 76%, reminiscent of the homology found between hNMT-1 and hNMT-2. Thus, most of the distinguishing features between the primary structures of NMT-1 and NMT-2 have been conserved from mouse to human. Northern analysis demonstrated that most human and mouse tissues concurrently express both NMTs, indicating that a functional difference between the two enzymes is not likely rooted in restricted tissue distribution profiles (data not shown).

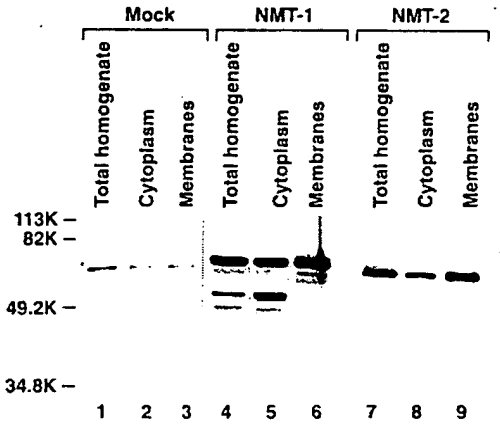

FIG. 3. **Western blot analysis of human NMT-1 and NMT-2 proteins expressed in COS-7 cells.** *Lanes 1–3*, mock-transfected COS-7 cell extracts; *lanes 4–6*, NMT-1-transfected COS-7 cell extracts; *lanes 7–9*, NMT-2-transfected COS-7 cell extracts. Each lane contains 10 μg of protein. Four immunoreactive NMT-1 isoforms (*lane 4*) of apparent sizes of 68, 56, 55, and 49 kDa were found in NMT-1-transfected cells (*lane 4*), while a single 65-kDa NMT-2 protein was identified in NMT-2-transfected cells (*lane 7*). Note the presence of weakly immunoreactive 67- and 64-kDa proteins in mock-transfected cells (*lanes 1* and *3*). These proteins may represent endogenous NMTs.

Expression of hNMTs in COS-7 Cells—To compare the hNMT-1 and hNMT-2 proteins further, each NMT cDNA was cloned into the eukaryotic expression vector pcDNA3 and transiently transfected into COS-7 cells. COS-7 cells expressing either hNMT-1 or hNMT-2 showed significantly higher levels of NMT activity than mock-transfected cells (Table I). Several peptides were tested as substrates for each NMT to gain a preliminary understanding of the relative substrate selectivities of each enzyme. Both hNMT-1 and hNMT-2 myristoylated peptide substrates corresponding to the N termini of cAMP-dependent protein kinase (PKA), Src kinase, and Abl kinase (Table I). Analysis of the initial reaction rates revealed that the enzymes shared similar substrate selectivities, with the notable exception that hNMT-2 showed a greater relative prefer-

ence for the Src peptide substrate. Neither NMT myristoylated either 1) a control peptide in which the N-terminal glycine from the PKA peptide was removed or 2) an internal peptide from the tumor necrosis factor α precursor protein, EEALPKKTGGPQGSR, which is myristoylated on lysine residues (32).

Affinity-purified antibodies generated against a GST fusion protein of hNMT-1 were used to characterize by Western blotting the expression of each NMT in COS-7 cells (Fig. 3). hNMT-1 and hNMT-2 appeared as primarily single protein bands of 68 and 65 kDa in size, respectively (Fig. 3, *lanes 4* and *7*). While these apparent sizes for hNMT-1 and hNMT-2 were larger than their predicted molecular masses (about 57 kDa for each enzyme), previous work has indicated that NMTs migrate anomalously large on SDS-polyacrylamide gel electrophoresis (19). Interestingly, three additional isoforms of hNMT-1 were also identified in transfected COS-7 extracts (apparent sizes of 49, 55, and 56 kDa; *lane 4*), while no additional forms of hNMT-2 were observed (*lane 7*). The multiple hNMT-1 proteins were likely the result of *in vivo* processing events, as an equivalent hNMT-1 protein profile was identified in transfected COS-7 cells harvested directly into standard SDS gel loading buffer (data not shown). Mock-transfected COS-7 cells showed weakly immunoreactive 64- and 67-kDa proteins that may represent endogenous NMTs (*lanes 1–3*). Separation of COS-7 extracts into cytoplasmic and membrane fractions revealed that hNMT-2 and the 68-kDa isoform of hNMT-1 appeared significantly in both the cytoplasmic and membrane fractions (*lanes 5, 6, 8,* and *9*), while the 49-, 55-, and 56-kDa isoforms of hNMT-1 were predominately cytoplasmic (*lane 5*). The majority of each NMT enzyme activity was found in the cytoplasm (85 and 75% for hNMT-1 and hNMT-2, respectively). However, such relative enzyme activity comparisons should be interpreted with caution, given that membrane associated inhibitory factors have been shown to reduce the apparent activity of membranous NMTs (33). Finally, considering that hNMT-1 has recently been shown to target to ribosomes (19), the membranous NMTs identified here may in fact reflect association of these proteins with ribosomes that are themselves bound to membranes.

Conclusions—The isolation and characterization of a second family of mammalian *N*-myristoyltransferases has profound implications on both the *in vivo* regulation and pharmaceutical targeting of protein myristoylation. The strict conservation of primary structure identified within both NMT-1 and NMT-2 families suggests unique functions for each enzyme *in vivo*. At the mRNA level, both enzymes were identified in most human and mouse tissues examined, while a preliminary analysis of hNMT-1 and hNMT-2's substrate selectivity demonstrated that the enzymes have similar, but distinguishable, substrate preferences. One difference observed between hNMT-1 and hNMT-2 was that only hNMT-1 was processed to lower molecular weight isoforms upon expression in COS-7 cells. The potential biological significance of these hNMT-1 isoforms remains to be determined, but it is intriguing to speculate that in different cell types the extent of NMT processing could vary, allowing for the generation of distinct NMT protein profiles.

Given the central role of myristoylation in facilitating cellular transformation (6) and viral propagation (9), NMTs stand as an attractive potential drug target. The discovery of a second family of mammalian NMTs allows for a reexamination of the part that each NMT plays in supporting tumorigenesis and/or viral infectivity. An exciting possibility arises for selective pharmaceutical intervention if certain tumors or viruses require the activity of primarily one type of NMT. In this regard, NMT activity and protein levels have been found to increase severalfold in human colorectal tumors (14, 15). Through the generation of NMT-1- and NMT-2-specific antibodies, the identification of the up-regulated NMT(s) in these tumors should be possible. Such NMT-1- and NMT-2-specific reagents in union with further examinations into the catalytic properties and cellular regulation of each enzyme should provide significant insights into the *in vivo* role of protein myristoylation in both normal and disease states.

Acknowledgments—We thank J. Rosenblum and M. Patricelli for critical reading of the manuscript.

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